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Synthesis of BODIPY-labeled alkylphosphocholines with leishmanicidal activity, as fluorescent analogues of miltefosine

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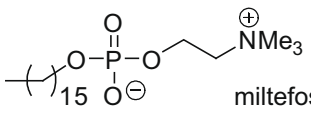
ABSTRACT

Two general synthetic methods are described, by which the highly fluorescent and photostable BODIPY group can be inserted in and aligned with the alkyl backbone of linear lipids. These methods have been used to prepare strongly emitting analogues of the leishmanicidal drug miltefosine, in which the antiparasite activity in vitro of the original drug is preserved.

Miltefosine (hexadecylphosphocholine, MT) was initially developed as an anticancer drug,[1] although MT largest current application is driven by its potent and selective antileishmanial effect.[2–4] In fact, MT became quite recently the first successful oral drug registered for the treatment of visceral leishmaniasis,[5] a fatal disease if untreated, as well as of other forms of this neglected, widespread tropical infection.[6,7] The molecular mechanism of the surprising antiparasitic activity of MT is far from being understood, in spite of its clinical relevance.[8,9] We recently synthesized a thiolated analogue of the drug,[10] in an effort to isolate putative therapeutic targets of MT by affinity chromatography. In addition to these techniques, medium[11] and high-resolution[12] fluorescence-based imaging methods might be used to determine the uptake, subcellular localization and many other important insights of the drug–parasite interaction.



miltefosine (MT)

The success in the application of these optical techniques is largely dependent on the availability of emitting drug analogues in which the biological activity remains essentially unchanged. To fulfill these requirements, we recently produced the first generation of fluorescent bioactive analogues of MT by tethering an ω-phenylpolyene group to the alkyl chain of the drug,[13] iterating a strategy used before to study the subcellular distribution of edelfosine, a chemically related glycerophospholipid with anticancer activity.[14] Despite of the less than ideal spectroscopic properties of the phenylpolyene group, such as low emission yield and UV excitation, these analogues provided important mechanistic information on the antiparasite effects of MT.[13] Nevertheless, to increase the spatial resolution of live-cell imaging experiments and the extent of the recording time, the brightness (absorption coefficient × emission yield), the color range and the photochemical and chemical stability of the fluorescent group attached to the MT structure will require a large improvement.

Here, we report an alternative way to greatly enhance the spectral properties of the emitting label, by the synthesis of three MT analogues in which a 4,4-difluoro-4-bora-3*a*,4*a*-diaza-*s*-indacene (BODIPY) fluorescent group has been inserted in, and aligned with, the drug alkyl chain. The potent antileishmanial activity of the parent drug has been preserved in the emitting analogues.

Previous studies on structure–antiparasite activity relationship for alkylphosphocholines[2,15–17] point to a preferred location of the fluorescent group at the extreme of a saturated alkyl chain, leaving intact the essential phosphocholine group, and with a total length of the lipophilic part close to that of a C16–C18 polymethylene chain. Moreover, because of the size of MT and of its

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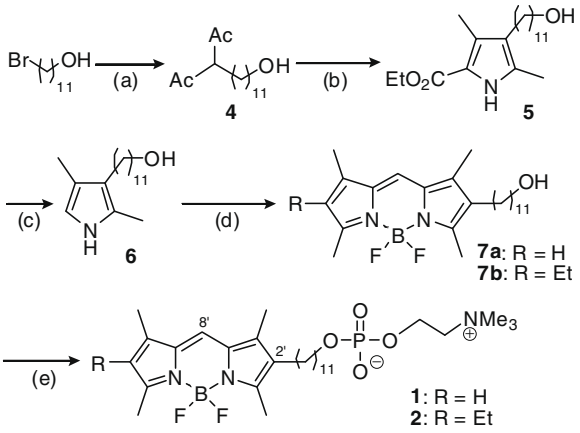
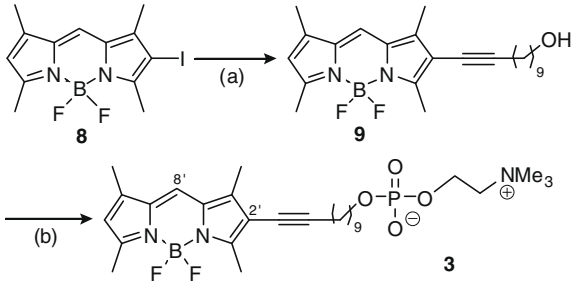
amphipathic properties, a small lipophilic fluorophore should be preferred. The previously obtained ω-phenylpolyene analogues met these conditions and presented high antiparasite activity, together with modest spectroscopic properties.[13] In the second generation of analogues presented here, both the phosphocholine head-group and the saturated alkyl chain are also maintained, but the emitting group is now the lipophilic BODIPY fluorophore. This optical label absorbs and emits in the visible range at convenient wavelengths for biological experiments, with high brightness and much-improved photostability.[18,19] In order to mimic the linear conformation of the original drug, the alkyl chain must be linked to the 2-position of the BODIPY group. The introduction of substituents at this position can be carried out through two methods[19]: (1) synthesis of the corresponding 2-substituted dipyrromethene intermediate from a pyrrole with the adequate substituent, by condensation of an α-H-pyrrole with an α-keto- or α-formylpyrrole; and (2) electrophilic substitution reactions (iodination, bromination) at position 2 of a 2-H-BODIPY, and further steps up to the final target structure. Both alternatives have been used in this work.

An analogue with the BODIPY group at the terminal position of the alkyl chain (**1**), and the same molecule tethered with an ethyl group (**2**), were selected as target compounds (Scheme 1), as well as a more rigid structure (**3**), with a triple bond conjugated with the BODIPY group (Scheme 2). In this way, analogues with similar size but different absorption and emission properties and, likely, different bioactivities could be prepared.

The synthesis of analogues **1** and **2** was carried out in two steps (Scheme 1): (1) the condensation of the hydroxyundecyl pyrrole **6** with 3,5-dimethyl-2-formyl-pyrrole or 3,5-dimethyl-4-ethyl-2-formylpyrrole, respectively, in the presence of phosphorous oxychloride (MacDonald condensation)[20] yielded a dipyrromethene



Scheme 1. Synthesis of MT analogues **1** and **2**. Reagents and conditions: (a) acetylacetone (10 equiv), K_2CO_3, 18-c-6, acetone, reflux, 24 h, 82%; (b) ethyl acetoacetate, $NaNO_2$, H_2O, AcOH, 5 °C, 3 h, then room temp., 14 h, add **4** (1 equiv) and Zn dust, 65 °C, 1 h, 58%; (c) NaOH, EtOH–H_2O, reflux, 3 h, 44%; (d) 1 equiv of 3,5-dimethyl-2-formyl pyrrole (for **7a**) or 3,5-dimethyl-4-ethyl-2-formyl pyrrole (for **7b**), $POCl_3$, CH_2Cl_2, Ar, 5 h, then DIPEA, $BF_3{\cdot}OEt_2$, 20 min, 52% (**7a**), 40% (**7b**); (e) 2-chloro-1,3,2-dioxaphospholane-2-oxide (2 equiv), Me_3N, MeCN, Ar, pressure tube, 78 °C, 3 h, then 80 °C, 4 h, 38% (**1**), 35% (**2**).



Scheme 2. Synthesis of the MT analogue **3**. Reagents and conditions: (a) undec-10-in-1-ol (1 equiv), $PdCl_2(PPh_3)$, CuI, phenol, *n*-Bu_4NI, DIPEA, DMF, room temp., 3 h, 74%; (b) 2-chloro-1,3,2-dioxaphospholane-2-oxide (2.1 equiv), Me_3N, MeCN, Ar, pressure tube, 78 °C, 3 h, then 80 °C, 4 h, 25%.

Table 1
Spectroscopic properties of fluorescent BODIPY analogues of miltefosine: wavelength of maximum absorption (λ_a) and fluorescence emission (λ_f), molar absorption coefficient (ε_{max}) and fluorescence quantum yield (Φ_f). T = 22 °C.

Compound	Solvent	λ_a (nm)[a]	ε_{max} (M^{-1} cm^{-1})	λ_f (nm)[a]	Φ_f[b]
1	EtOH	517		525[c]	
	DMSO	517	71,100	526[c,d]	0.93[c,d]
2	MeOH	527	82,000	536[e]	0.94[e]
	EtOH	529	79,000		
	DMSO	529	79,000		
3	EtOH	533	61,300	553[e]	0.51[e]

[a] ±1 nm.
[b] ±10%.
[c] λ_{exc} = 490 nm.
[d] In MeOH.
[e] λ_{exc} = 502 nm.

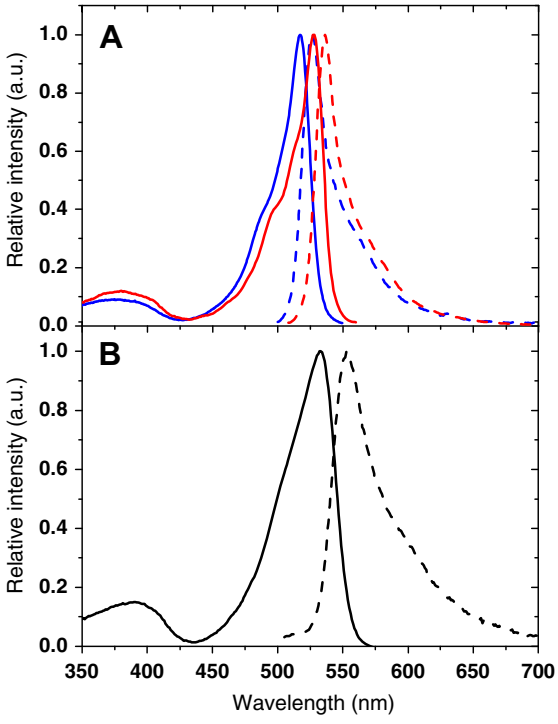


Figure 1. Normalized absorption (—) and corrected fluorescence (---) spectra of analogues **1** (blue) and **2** (red) in DMSO (A), and **3** in EtOH (B). Concentration 10^{-5}–10^{-6} M, T = 22 °C.

Table 2
In vitro antileishmanial activity[a] of BODIPY analogues of miltefosine (MT) **1** and **2** on *Leishmania donovani* promastigotes.[13,27]

Compound	LD_{50} ± SE/μM
MT	6.5 ± 0.2
1	5.2 ± 0.2
2	4.3 ± 0.1

[a] Drug concentration required to inhibit 50% parasite proliferation.

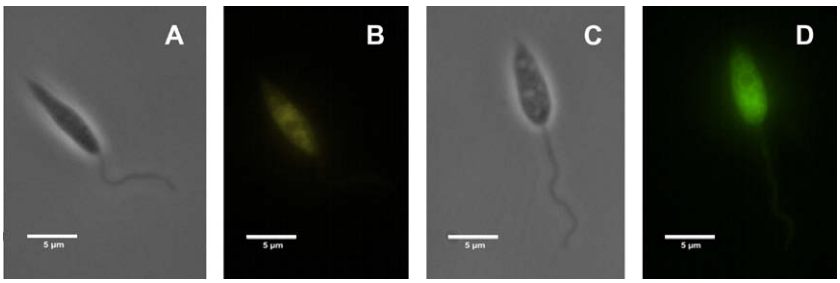


Figure 2. Live-cell light transmission (A and C) and fluorescence (B and D, false color) micrographs showing the specific incorporation of BODIPY analogues of miltefosine to *Leishmania donovani* promastigotes. (A and B) analogue **2**; (C and D) analogue **1**; bar: 5 μm.[28]

intermediate that was converted in situ into the corresponding BODIPY compounds **7a** and **7b** by reaction with boron trifluoride diethyl etherate in the presence of *N,N*-diisopropylethylamine (DIPEA)[21]; (2) the phosphocholine group was introduced in alcohols **7a** and **7b** by reaction with 2-chloro-1,3,2-dioxa-phospholane-2-oxide and trimethylamine, as detailed elsewhere.[10] Pyrrole **6** was synthesized in three steps: (1) alkylation of acetylacetone with 11-bromoundecanol in the presence of 18-crown-6, yielding alcohol **4**; (2) condensation of **4** with ethyl acetoacetate under Johnson–Knorr conditions,[22,23] giving rise to pyrrole **5**; and (3) elimination of the methoxycarbonyl group in ester **5** by hydrolysis and decarboxylation.[24]

The synthesis of analogue **3** was carried out in two steps by a different method (Scheme 2): (1) a Sonogashira–Hagihara cross-coupling reaction[25] between the 2-iodo-BODIPY compound **8** synthesized as described[26] and undec-10-yn-1-ol, in the presence of dichlorobis(triphenyl-phosphine)palladium(II) and copper(I) iodide as catalyst, afforded the acetylenic alcohol **9**; and (2) the reaction of **9** with 2-chloro-1,3,2-dioxaphospholane-2-oxide and trimethylamine, as above, yielded the expected analogue **3**.

These two methods allowed the preparation of the fluorescent analogues by formation of a carbon–carbon bond, thus avoiding the introduction into the molecule of functional linkers with potentially reactive polar groups, such as amide, ester, ether, etc. Moreover, the extended conformation of the original apolar chain in MT is well preserved in the analogues. In this way the impact of the structural modifications on the bioactivity is minimized, because the fluorophore is aligned with the polymethylene chain by its attachment at the BODIPY 2-position. In fact, a miltefosine analogue similar to **1** but with the BODIPY group attached through the 8-position did not show leishmanicidal activity (results not shown). These synthetic methods can be extended to introduce the BODIPY fluorophore at different positions along the alkyl chain of MT, as well as at similar chains of other lipids of biological relevance.

Analogues **1**, **2** and **3** show excellent spectroscopic properties for the intended application as probes of antiparasite activity (Table 1): high molar absorption coefficient in the visible range, intense green (**1**), yellow (**2**) and orange (**3**) emission, with high quantum yield close to unity for **1** and **2** and high photostability. The spectral properties of the MT analogues **1**, **2** and **3** differ very little from those of the precursor alcohols **7a**, **7b** and **9**, respectively (results not shown), indicating the absence of electronic interaction between the fluorophore and the phosphocholine head-group. In analogue **2**, the ethyl group at position 6 of the BODIPY group shifts 12 nm to the red the wavelength of the maximum absorption, compared to **1**. The presence of a conjugated triple bond in analogue **3** does not diminish the good photostability of the emitting group, although other spectral properties changed noticeably. Thus, the main absorption band of **3** becomes broader, its absorption and emission maxima are red-shifted 16 and 28 nm, respectively, with regard to **1** (Fig. 1), and the fluorescence yield is lower (0.51).

The antiparasite activity of the fluorescent analogues **1** and **2** matched very well that of the original miltefosine drug. The in vitro leishmanicidal activity of these compounds (analogue **3** is yet to be tested) against promastigote forms of *Leishmania donovani* was in the 10^{-6} M range, that is, comparable to that of MT under similar conditions (Table 2).[27] In addition, the emitting analogues were recognized by an uptake system of *Leishmania*, giving rise to a fast, specific fluorescent staining of the whole living parasite (Fig. 2).[28]

In conclusion, it is shown here two general synthetic methods by which the strongly fluorescent BODIPY group can be introduced in the non-polar part of a large variety of lipids, aligned with the alkyl backbone and with minimal perturbation of the amphipathic properties of the original molecule. These methods were used to prepare the first highly fluorescent in the visible range and photostable analogues of the leishmanicidal drug miltefosine, with in vitro antiparasite activity comparable to that of the original alkylphosphocholine.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.10.089.

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27. Promastigotes of *Leishmania donovani*, strain MHOM/ET/67/L82R40 kindly provided by Prof. S.L. Croft (London School of Tropical Hygiene and Medicine), were incubated (2×10^6 cells mL^{-1}, initial inoculum) 72 h in a 96-microwell plate, in the presence of each fluorescent analogue or miltefosine, assaying the 0.5–50 μM concentration range. Proliferation was quantified from the extent of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction by the parasites, as determined by the absorbance change at 595 nm in a Bio-Rad 450 Microplate Reader.
28. Samples for light transmission or fluorescence microscopy were prepared by incubating promastigotes (2×10^7 cells mL^{-1}) in growth medium in the presence of analogue **1** or **2** (7 μM) for 4 h at 25 °C. The parasites were then washed three times with 1 mL of phosphate saline containing 10 mg mL^{-1} of bovine seroalbumin (SIGMA, fatty-acid free). Living parasites were imaged in a Zeiss Axioplan Universal epifluorescence microscope fitted with the fluorescein filter set (Chroma, Battleboro, VT), excitation bandpass 465–495 nm and emission bandpass 515–550 nm. Images were recorded with a Leica DFC 350FX digital camera.